Measurement and subcellular distribution of choloyl-CoA synthetase and bile acid-CoA:amino acid *N*-acyltransferase activities in rat liver

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Abstract An improved method for assaying choloyl-CoA synthetase activity (E.C. 6.2.1.7) and two methods for specific measurement of bile acid-CoA:amino acid *N*-acyltransferase activity (E.C. 2.3.1) are described. The methods are shown to be reproducible, linear with respect to time and enzyme protein, and result in estimates of enzymic activity that conform to the theoretical stoichiometry of the individual reactions. Utilizing these methods, the subcellular distribution of the rat liver enzymic activity catalyzing the formation of glycine and taurine conjugates of bile acids is shown. Choloyl-CoA synthetase is associated with the microsomal membranes and bile acid-CoA:amino acid *N*-acyltransferase activity with the postmicrosomal supernatant. No significant amino acid *N*-acyltransferase activity is present in the lysosome fraction. These studies provide methods that will permit further study of the individual enzymic reactions involved in the intrahepatic conjugation of bile acids with amino acids.

Supplementary key words bile acids · conjugation · amino acids · enzyme assays

Several authors have published evidence indicating that the intrahepatic synthesis of the amino acid conjugates of bile acids is the result of two independent enzymic reactions with a bile acid coenzyme A thioester intermediate (1–3).

$$\text{bile acid} + \text{ATP} + \text{CoASH} \xrightleftharpoons{Mg^{2+}} \text{bile acid-CoA} + \text{AMP} + \text{PPi} \quad \text{Eq. 1.}$$

$$\text{bile acid-CoA} + \text{amino acid} \rightleftharpoons \text{bile acid-}N\text{-amino acid} + \text{CoASH} \quad \text{Eq. 2.}$$

Subsequent in vitro investigation of enzymic conjugation of bile acids has been exclusively dependent on methods in which the above two reactions are coupled and the conjugation of added bile acid with glycine and taurine is measured. Despite the use of radiolabeled substrates, these methods require prolonged incubations during which the rate of product formation is nonlinear with respect to either time or hepatic protein (3, 4). Although Bremer (3) introduced a technique for independent assay of the choloyl-CoA synthetase reaction (E.C. 6.2.1.7, Eq. 1) the method depends upon the conversion of the CoA thioester to the hydroxamate and is relatively insensitive. A more sensitive method for measuring choloyl-CoA synthetase activity has recently been described by Polokoff and Bell (5). Bile acid-CoA:amino acid *N*-acyltransferase activity (E.C. 2.3.1, AAT) shown in Eq. 2 has not been measured directly. The activity of this step has been inferred from parallel experiments in which the formation of hydroxamates and of amino acid conjugates were measured. However, dependence of AAT activity on coexistent choloyl-CoA synthetase activity prevented independent study of the second step.

The lack of an independent assay of AAT activity has prevented direct determination of the subcellular distribution of the enzyme protein(s) catalyzing this step. Bremer and Gloor (6) noted in rats that, while both the microsome fraction and postmicrosome supernatant were required for conjugation of bile acids, boiled supernatant could substitute for native supernatant. They therefore concluded that the enzymic proteins responsible for both steps were present in microsomes.

However, Siperstein and Murray (1) achieved formation of taurocholic acid by incubating enzymically synthesized choloyl-CoA with taurine in the presence of postmicrosome supernatant, suggesting that AAT was in the soluble fraction. In later studies, Schersten, et al. (7) found that conjugation of bile

Abbreviations: AAT, bile acid-CoA:amino acid *N*-acyltransferase; PES, phenazineethosulfate; DCPIP, 2,6-dichlorophenolindophenol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, morpholinopropanesulfonic acid; TKMS buffer, 50 mM Tris-HCl, pH 7.8, 25 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose; A_{232}, absorbance at 232 nm.

acids by human liver subcellular fractions was maximal when the lysosome fraction was combined with microsomes. Since these authors observed no increase in choloyl–hydroxamate formation under these conditions, they concluded that AAT fractionated with lysosomes. Resolution of these three conflicting observations has never been attempted using a specific assay for AAT activity.

Recently, we described the chemical synthesis of substrate quantities of biologically active coenzyme A thioesters of bile acids (8). The availability of these intermediates has permitted the development of specific methods to assess AAT activity independent of choloyl-CoA synthetase activity. This paper describes these methods as well as a method of measuring choloyl-CoA synthetase activity and, using these specific methods, reports the subcellular localization of the enzymic activities catalyzing the individual steps of bile acid conjugation in the rat.

MATERIALS AND METHODS

Chemicals

Disodium ATP and lithium coenzyme A were obtained from P-L Laboratories, Milwaukee, WI. PES and DCPIP were purchased from Sigma Chemical Company, St. Louis, MO. 3-α-Hydroxysteroid dehydrogenase was obtained from Worthington Biochemicals, Freehold, N.J. Carboxylated ^{14}C-labeled bile acids were obtained from Mallinckrodt, St. Louis, MO. Source and purity of the unlabeled bile acids and the coenzyme A thioesters of bile acids have been previously described (8). ^{14}C-Labeled bile acid-CoA derivatives were synthesized by the same method and exhibited a final sp act of approximately 20 μCi per μmol. Defatted bovine serum albumin was prepared according to the method of Chen (9). Other chemicals were of analytic quality and were obtained from commercial sources.

Analytic methods

Except as noted, variation in the means of repeated samples is expressed as ±SEM. CoASH was estimated according to the method of Beutler, Duron, and Kelly (10). Determination of enzymically synthesized amino acid conjugates of bile acids was performed as previously described (8). Protein was estimated by the biuret method following solubilization with deoxycholate (11).

Subcellular fractionation was performed on liver from male Sprague-Dawley rats (175–225 g). Homogenization and fractionation were carried out in 300 mM sucrose and 5 mM MOPS buffer, pH 7.2, as described by Hoppel and Tomec (12) except that microsomes were isolated in a Beckman model L 2-65B ultracentrifuge, using a model 50 rotor. Subcellular fractions were assayed for marker enzyme activity by published methods (11–13).

Choloyl-CoA synthetase activity. The assay system is derived from that reported by Suzue and Marcel (14) for long chain acyl-CoA ligase and analogous to the method recently reported by Polokoff and Bell (5). The complete system contained 5 mM ATP, 5 mM $MgCl_2$, 0.2 mM bile acid, 0.2 mM CoASH, 50 mM NaF, 100 mM sodium phosphate, pH 7.5, 5–10 × 10^5 cpm of [^{14}C]bile acid, and enzyme protein in a total volume of 0.5 ml. The concentrations of substrates and cofactors given above were optimal as determined by individual concentration–activity curves.

The assay mixture minus enzyme was brought to 37°C in a water bath and the enzyme was added. After 0.5–5 min, the reaction was stopped by addition of 0.5 ml of ice-cold methanol. After an additional 5 min on ice, the test tubes were covered with paraffin film and the medium was centrifuged to precipitate the protein. Aliquots (0.7 ml) of supernatant were removed and added to 0.5 ml of 6% (v/v) perchloric acid. This was extracted twice with 10 ml of dry ethyl ether, resulting in the removal of more than 99% of the unreacted bile acid (**Table 1**). The aqueous phase containing the ether-insoluble bile acid-CoA product was suffused for 20 sec with dry nitrogen gas to remove the ether residue. Aliquots of the remaining aqueous phase were neutralized with 50 μl of 11.5 N ammonium hydroxide and were counted in a liquid scintillation system at 5°C (50% efficiency). To correct for quenching, an aliquot of the radiolabeled bile acid originally added to the reaction mixture was used as an internal standard.

TABLE 1. Requirements of the choloyl-CoA synthetase assay

Addition	Product Formed	
	cpm	*nmol*
Complete system[a]	1494	4.11
minus ATP	200	0
minus $MgCl_2$	340	0.42
minus CoASH	217	0
minus NaF	1418	3.85
minus microsome protein	206	0
incubated at 0°C	216	0

[a] The complete system was incubated at 37°C for 5 min in the presence of 200 μM cholic acid, 9 × 10^4 cpm [^{14}C]cholic acid, and 0.25 mg of rat liver microsome protein. Other conditions are as described in the text. Values shown are the averages of duplicate determinations.

Control reactions were maintained on ice during the incubation period and carried through the remainder of the assay in parallel with the experimental assay. The variation of duplicate determinations was 5 ± 2%.

AAT assays. Two methods were used. The first measured amino acid-dependent, enzymic release of CoASH at 30°C by recording the rate of reduction of the blue dye, DCPIP, in the presence of PES (15, 16). The reaction mixture contained 25 mM HEPES buffer, pH 7.2, 50 μM bile acid-CoA, 50 μM DCPIP, 20 μM PES, 1 mg of defatted bovine serum albumin, enzyme, and 20–80 mM taurine or 40–100 mM glycine in a final volume of 1 ml. All reagents except enzyme and amino acid were combined and warmed to 30°C for 2 min. Enzyme was added and dye reduction was monitored at 30°C for 3 min. Amino acid, previously brought to pH 7.2 and prewarmed to 30°C, was the final addition initiating the reaction. Experiments were run in sets of four parallel incubations: two contained the complete reaction mixture, in one the enzyme was omitted, and one lacked bile acid-CoA substrate. In the latter two incubations, volume was adjusted to 1 ml with water.

The rate of reaction was controlled by adjusting the amount of enzyme protein added so that the complete system had a reaction rate between 0.5 and 1.0 nmol DCPIP reduced per min. Following the addition of amino acid, there was a 15–20 sec equilibration period after which the rate of loss of absorbance became linear with time and remained so for up to 4 min. At the end of this time, 4 nmol of DCPIP had been reduced. Enzymic activity was optimal at the concentrations of DCPIP and PES noted above. At higher concentrations inhibition was frequently noted. The variation of the mean of duplicate determinations was 3 ± 1%.

The reaction mixtures lacking either enzyme or bile acid-CoA substrate served as controls. The rate of dye reduction in the more active of the two controls was subtracted from the mean of the two complete incubations. In all situations, the rate of dye reduction in the complete incubation was greater than four times the rate in the more active control. Rapid dye reduction was occasionally noted prior to addition of amino acid to the otherwise complete incubation mixture. In these instances, an additional control was used in which water replaced the amino acid in an otherwise complete mixture; this latter control corrected for choloyl-CoA hydrolase activity (17).

The second assay measured AAT activity by recording the amino acid-dependent enzymic loss of the thioester bond estimated from the rate of change in absorbance at 232 nm (A_{232}). The previously determined millimolar extinction coefficient of 4.12 was utilized (8). The complete system was incubated at 30°C and contained 50 mM potassium phosphate buffer, pH 7.2, 50 μM bile acid-CoA thioester, and amino acid at concentrations noted above. Controls similar to those utilized in the DCPIP reduction method were employed. Change in absorbance was linear after 15 sec and remained constant for up to 2 min. The rate of reaction was adjusted by varying the amount of protein added so the final rate was between 2 and 7.5 nmol per min, a maximum of 15 nmol of CoA thioester having been consumed during the linear period. The reproducibility of this assay was equivalent to that noted with the former method.

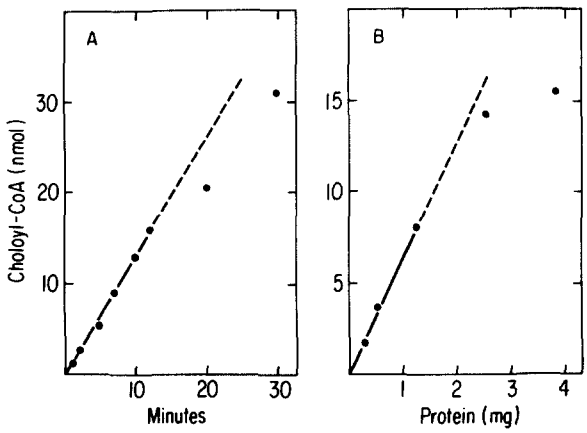

Fig. 1. Variation in choloyl-CoA synthetase activity with time of incubation and protein content. Choloyl-CoA synthetase activity was measured as described in the text. In Fig. 1*A*, 0.26 mg of rat liver microsome protein was added. Time of incubations shown in Fig. 1*B* was 1 min.

RESULTS

Choloyl-CoA synthetase assay

The requirements for the assay are shown in Table 1. Product formation with the complete system was linear with time up to 12 min and with added protein between 0.1 mg and 1.2 mg of crude microsome fraction (**Fig. 1**). The time of incubation and protein content of each assay were adjusted so that the ratio of the cpm in an experimental assay to its control was at least 3. Under these conditions, the lower limit of product formation that could be reliably detected was between 0.3 and 0.5 nmol. The addition of cysteine or nicotinamide, both used by Bremer et al. (2, 6) in previous assays, resulted in either no advantage in radioactive product formation or slight inhibition. Sterol carrier protein

prepared according to the method of Ritter and Dempsey (18) was likewise without effect in this assay.

The assay is based on the assumption that, at acid pH, the bile acid-CoA thioesters are insoluble in ether and therefore remain in the water phase (19). This has recently been confirmed with [^{3}H]choloyl-CoA by Polokoff and Bell (5). In the present assay, when 0.6–3.0 nmol of [^{14}C]choloyl-CoA or [^{14}C]chenodeoxycholoyl CoA was added to the complete system in place of [^{14}C]bile acid, 101 ± 4% of the added radioactivity was accounted for in the final aqueous aliquot. Recovery of radioactivity in these experiments was independent of initial protein concentrations between 0.4 mg and 2.0 mg. As noted in **Table 2,** radioactive product formation calculated in this manner is stoichiometric with respect to CoASH consumption.

AAT

The DCPIP reduction method was the more sensitive of the two methods but has certain limitations. Product formation was linear with protein only up to a rate not exceeding 1 nmol per min. This limitation is independent of the sp act of the enzyme protein (**Fig. 2**). Consequently, we chose to adjust the protein added to the reaction cuvette such that the rate of DCPIP reduction fell between 0.5 and 1.0 nmol per min regardless of the sp act of the protein used. In addition, DCPIP reduction can occur as a consequence of oxidation of substances other than the CoASH released by AAT. Such situations were occasionally encountered with crude protein fractions and especially when glycine was utilized as the amino acid acceptor. Nonspecific reduction of DCPIP has not exceeded a rate of 0.3 nmol per min under the usual experimental conditions. This problem can be surmounted by use of a control incubation in which the bile acid-CoA substrate is omitted. Even in larger scale incubations with high control rates, the change in absorbance of DCPIP is stoichiometric with formation of bile acid conjugates (Table 3).

Although the A_{232} method was less sensitive than DCPIP reduction, loss of absorption at 232 nm was linear over a greater range of protein concentrations and nonspecific side reactions were not a problem (Fig. 2). The assay, however, could be run only with a limited number of buffers. We chose phosphate buffer because of its minimal ultraviolet absorption at 232 nm. Use of Tris and HEPES buffers resulted in a higher sp act, but the buffers themselves exhibited considerable absorption at 232 nm, resulting in high background absorbance. In the presence of phosphate buffer, loss of absorption at 232 nm was stoichiometric with conjugation (**Table 3**). As determined from the slopes of the lines in Fig. 2, the sp act of the partially purified AAT was 49 nmol/min per mg with the A_{232} method and 51 nmol/min per using the DCPIP method, indicating agreement between the two methods.

TABLE 2. Partial stoichiometry of the choloyl-CoA synthetase assay

Measurement	nmol
[^{14}C]Choloyl-CoA formed[a]	55.0 (53.6–56.5)
CoASH consumed	54.2 (52.9–57.4)

[a] Incubation was for 30 min in the presence of 400 μM cholic acid, 400 μM CoASH, and 1.8 mg of rat liver microsome protein. Other conditions are as described in the text. Radioactive product and CoASH were determined on aliquots of parallel incubations. Values are means and range of four determinations.

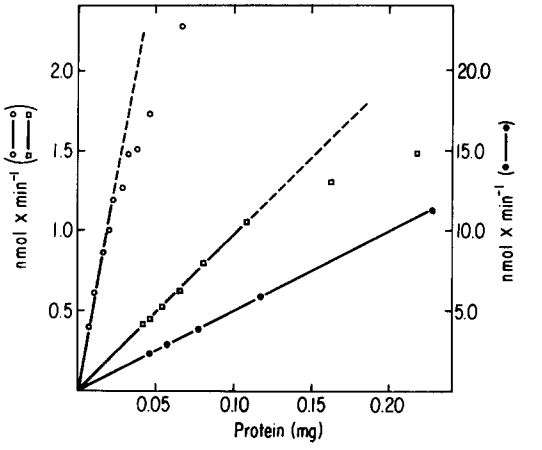

Fig. 2. Relationship between the measured rate of AAT activity and protein. Methods are as described in the text. 20 mM taurine was the amino acid acceptor. DCPIP reaction was measured with crude rat liver homogenate (□ —— □), sp act 9.8 nmol/min per mg, and with the fraction of final supernatant that precipitated following the addition of solid ammonium sulfate to a final concentration of 2.3 M (○ —— ○), sp act 51 nmol/min per mg. This latter protein was also used in the A_{232} system (● —— ●). The ordinate for the A_{232} method is indicated on the right.

Subcellular distribution of bile acid conjugation enzymes

The distribution of the bile acid conjugation enzymes was compared to that of marker enzyme activity of fractions of rat liver homogenate (**Table 4**). Marker activity, recovery, and distribution among the fractions were comparable to those observed by others (12, 20). Although there was contamination of the microsome fraction by lysosomal acid phosphatase, the acid phosphatase sp act was highest in the lysosome fraction and was 1.4 times the acid phosphatase sp act in the microsome fraction.

TABLE 3. Comparison of spectrophotometric change and formation of bile acid conjugates for two methods of assaying AAT activity

Method		Time	Spectrophotometric Estimate			Conjugates Formed[c]
			Complete System[a]	Control[b]	Net	
		min	*nmol*	*nmol*	*nmol*	*nmol*
DCPIP reduction	a	10	467	124	343	348
	b	15	967	62	904	999
	c	5	440	49	391	445
	d	10	876	114	762	759
A_{232} reduction	e	10	988	49	939	1012
	f	10	983	48	935	912
	g	10	1268	0	1268	1367

[a] The conditions for the complete system were as follows. DCPIP reduction: 0.3 mM PES, 0.3 mM DCPIP, 0.3 mM deoxycholoyl-CoA, 20 mM taurine, 25 mM HEPES, pH 7.2, and 5 mg of defatted bovine serum albumin in 5 ml. A_{232} reduction: 50 mM sodium phosphate, pH 7.2, 0.3 mM chenodeoxycholoyl CoA and 20 mM taurine in 5 ml. Enzyme protein for the above reactions was prepared as follows. In experiments *a*, *b*, *e*, and *f*, 0.62 mg of that portion of rat supernatant fraction that precipitated at 2.3 M ammonium sulfate was used. Protein used in *b*, *e*, and *f* was from the same preparation, *a* was similarly prepared but from a different rat. Protein used in *c*, *d*, and *g* was 0.48 mg of rat supernatant that had been carried through a second ammonium sulfate fractionation. Protein used in *c* and *d* was from the same rat.

[b] Control reactions did not contain acyl-CoA substrate.

[c] Methods as in the text (8).

Choloyl-CoA synthetase activity was distributed with the microsome fraction as originally shown by Bremer and Gloor (6). Greater than 97% of this activity remained in the microsome pellet without loss of activity when freshly prepared microsomes were rehomogenized and recentrifuged in the presence of either 15 mM cesium chloride, 90 mM disodium EDTA (4.1 μg/mg microsome protein), or 10 mM sodium pyrophosphate, suggesting that choloyl-CoA synthetase activity is associated with the microsome membrane (21–24). There was no difference in the distribution of the activity when deoxycholic and chenodeoxycholic acids were used as substrate for the reaction.

TABLE 4. Intracellular distribution of bile acid conjugating enzymes and some marker enzymes in rat liver

Enzyme	Specific Activity[a]	Percent Recovery of Activity in Subcellular Fractions[b]					
		N	M	L	P	S	Total
Choloyl-CoA synthetase AAT[c]	0.156	3.8	4.4	2.3	84.3	0.4	95.2
glycine-dependent	15.4	14.3	3.8	5.2	26.0	41.2	90.5
taurine-dependent	7.14	11.3	3.6	4.0	24.6	52.2	96.0
Glucose-6-phosphatase	14.0	9.1	11.5	8.9	64.7	5.7	99.9
Lactate dehydrogenase	170	5.1	0.9	0.2	16.8	73.6	96.6
Acid phosphatase (total activity)	5.28	7.9	10.1	27.3	47.6	14.8	107.7
Acid phosphatase (free activity)	1.39	1.3	6.7	8.0	13.3	1.4	(30.9)
Glutamate dehydrogenase	12.9	14.0	79.1	4.3	10.6	2.4	110.4
Succinate dehydrogenase	1.78	11.0	72.5	6.4	7.8	0.3	98.0
5′-Nucleotidase	8.52	26.1	2.8	6.7	58.7	7.7	102.0
Protein	398.0	10.7	12.2	7.6	25.8	36.6	92.9

[a] Specific enzyme activity is expressed as μmol of substrate metabolized per g (wet weight) of liver, per minute. Free acid phosphatase activity is given as the percent of total acid phosphatase activity in the whole cell homogenate. Protein is expressed in mg per g (wet weight) of liver.

[b] Recoveries are calculated according to published methods (11, 20). Individual fractions are: *N*, nuclear; *M*, mitochondrial; *L*, lysosomal; *P*, microsomal; *S*, final supernatant.

[c] AAT activities were determined by the DCPIP reduction method as described in the text in the presence of 100 mM glycine or 80 mM taurine.

With the standard DeDuve fractionation, the majority of the glycine- and taurine-dependent AAT activity was distributed with the microsomes or with the final supernatant (Table 4). Substitution of deoxycholoyl-CoA and chenodeoxycholoyl-CoA as substrates resulted in similar distribution. The fraction of AAT activity that migrated with the microsome pellet was released into the supernatant following resuspension of the microsomes in the TKMS buffer described by Petermann and Pavlovec (25) (**Table 5**), suggesting that AAT activity is soluble and that its association with the microsome membrane was due to nonspecific, coulombic interaction (23, 26). When microsomes were initially prepared in this buffer, only 6% of the AAT activity distributed with the microsome pellet.

The relative distribution of AAT activity was not changed nor was latent AAT activity detected when the subcellular fractions were treated with polyoxyethylene ether (Triton X-100, 100 μg/mg protein), freezing and thawing, or dialysis for 2 hr against 1000 volumes of 1.3 M potassium chloride and 0.02 M potassium bicarbonate, pH 8.0. Recombination of individual fractions with the supernatant or microsome fractions did not result in stimulation of AAT activity.

DISCUSSION

The assay methods proposed in this work required relatively short incubation times during which product formation was linear with time. The advantage of preservation of linear production of product with time is evidenced by the fact that the sp act of choloyl-CoA synthetase with our assay was greater than 9 times that reported by Bremer (2) or Schersten (4) with comparable preparations and similar to that recently reported by Polokoff and Bell (5) utilizing an analogous assay. The present methods for the first time permit accurate measurement of each of the reactions of conjugation independently, thereby permitting specific study of the AAT reaction.

Using the present methods, the subcellular distribution of choloyl-CoA synthetase was the same as that reported by Schersten (4) in humans and Bremer and Gloor (6) and Polokoff and Bell (5) in rats. However, AAT activity in our studies initially appeared to exhibit a bimodal distribution, with major fractions of the total activity localized in both microsomal and supernatant fractions. The ease with which microsomal AAT activity can be solubilized independent of a major loss of protein from the microsomal pellet (Table 5) suggests that AAT activity is soluble and is nonspecifically adsorbed to microsomal membranes (23–27). Similar instances of nonspecific, coulombic adsorption of soluble enzymes to intracellular particles have been described for other enzyme proteins (24, 27–29).

Contamination of microsomal preparations with soluble AAT activity explains Bremer and Gloor's (6) finding that conjugation of cholic acid could be accomplished using only the microsome fraction. Comparison of the sp act of choloyl-CoA synthetase and AAT activities suggest that the synthetase reac-

TABLE 5. Release of microsome-bound AAT activity into supernatant following treatment of microsome fraction with TKMS buffer

Enzyme Activity	Fraction	Buffer: Sucrose–MOPS[a] Units	Sucrose–MOPS[a] Sp Act	TKMS Units	TKMS Sp Act
		nmol/min	*units/mg*	*nmol/min*	*units/mg*
Glycine–AAT	Supernatant	461	9.60	2181	31.7
	Pellet	1932	10.1	328	1.78
	Total	2393	(9.97)	2509	(9.92)
Taurine–AAT	Supernatant	275	5.73	1363	19.8
	Pellet	1407	7.33	206	1.12
	Total	1682	(7.10)	1569	(6.20)

[a] 300 mM sucrose, 5 mM MOPS, pH 7.2.

Equal aliquots of a microsome fraction prepared in sucrose–MOPS buffer as in Table 4 were resuspended in the indicated buffers and recentrifuged at 243 *g* for 30 min. Supernatant fractions from each were decanted and the pellets were resuspended in the buffer from which they were precipitated. Enzyme activity was measured by the DCPIP reduction method as described in the text. Data are the means of duplicate assays.

tion may be rate limiting in an in vitro coupled system. Minimal contamination of microsomes with AAT, therefore, would be sufficient to permit the conjugation of bile acids to proceed to completion.

The hypothesis of Schersten et al. (7) that AAT is lysosomal is based on the observation that conjugation of cholic acid, but not choloylhydroxamate formation, was enhanced when microsomes were incubated in the presence of lysosomes or lysosomal protein. Using our direct assays, we found no evidence of enrichment of AAT activity in the lysosome fraction nor was AAT activity distributed in synchrony with acid phosphatase activity as suggested by Schersten et al. Furthermore, recombination of lysosomes with microsomes or soluble fraction did not result in stimulation of AAT activity with either of the assay systems. We conclude, therefore, that the stimulation that Schersten et al. (7) observed may have been due to an effect of lysosomal enzymes on the incubation conditions of the coupled system rather than to the addition of AAT activity.

The AAT sp act in Table 4 reflect the concentration of amino acid used in the assays. At physiologic amino acid concentrations, the ratio of glycine-dependent to taurine-dependent AAT sp act was less than one. The amino acid concentrations used in the present work, while in excess of physiologic concentrations, were optimal with respect to the assay systems. These assays will permit study of the substrate kinetics of the AAT reaction and will be helpful in determining the extent to which AAT controls the pattern of bile acid conjugation in rats and other species.

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